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The overall goal in this proposal is to understand the mechanisms by which neurotoxicity and excitotoxicity destroy cells in the substantia nigra. Our hypothesis to test this idea is that c-JUN kinase (JNK), specifically the subtype JNK3, mediates degeneration of substantia nigra neurons after exposure to MPTP or glutamate excitotoxicity. Results in the first year of the project indicate that glutamate agonists at NMDA and kainate receptors increase the biological activity of JNK in the basal ganglia (cultured striatal neurons). The subtype JNK3 accounts for most of the JNK activity. Striatal neurons from JNK3 knockout mice lacked most of the biological activity of JNK after glutamate agonist treatment, and the size of the JNK responsible for the biological activity corresponded to JNK3. We also observed another, larger protein (complex) with JNK activity. In vivo studies revealed that the MPTP-induced neurotoxicity in wild-type mice was ameliorated in JNK3 knockout mice, but JNK1 and JNK2 offered even greater neuroprotection. Altogether, these year 1 results implicate JNK3 as the principal JNK target of glutamate stimulation in the striatum and JNK's as important mediators of MPTP induced neurotoxicity.

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FOREWORD

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N. Aronin

PI - Signature Date

ABSTRACT

The overall goal in this proposal is to understand the mechanisms by which neurotoxicity destroys cells in the substantia nigra. Our hypothesis to test this idea is that c-JUN kinase (JNK), or one of its isoforms, mediates degeneration of substantia nigra neurons after exposure to MPTP or glutamate excitotoxicity. In the first year, we examined mice for susceptibility to MPTP induced neurodegeneration. Our groups included mice treated with minimally 40 mg/kg of MPTP (10 JNK knockout and 4 wild-type) and mice receiving vehicle control (4 JNK and 3 wild-type). One week after the initial injection the mice brains were studied by immunohistochemistry for the presence of tyrosine hydroxylase labeled neurons in the substantia nigra. Results were as follows (per section of substantia nigra): vehicle-treated wild-type mice, 118±2 (SE); vehicle-treated JNK knockout mice, 133±2; MPTP-treated wild-type mice, 35±2; and MPTP-treated JNK knockout mice, 100±3. Statistical analysis of these preliminary data revealed a significant interaction between MPTP-treated wild-type vs MPTP-treated JNK ($p=0.016$) and vehicle-treated wild-type ($p=0.05$). These first studies provisionally indicate that one or several of the JNK isoforms might mediate the MPTP-induced death of tyrosine-hydroxylase neurons in the substantia nigra. We recognize that these preliminary results need to be extended with many more mice in each experimental group, particularly with use of additional mice JNK knockout mice for each JNK isoform. These studies will serve as the core of the experiments in year 2.

PROTECTIVE MECHANISMS AGAINST APOPTOTIC NEURODEGENERATION IN THE SUBSTANTIA NIGRA

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INTRODUCTION

Subject and purpose. Neurotoxins damage and kill neurons. Studies on neurotoxins can provide information on mechanisms in the pathogenesis of neurodegenerative diseases. Our overall goal in this study is to understand the mechanisms by which neurotoxicity and excitotoxicity destroy cells in the substantia nigra. Neurodegeneration in the substantia nigra causes Parkinson's disease.

Two classes of neurotoxins can be used to destroy neurons in the substantia nigra. MPTP administered intraperioneally kills substantia nigra cells, causing a rapid-onset, clinical Parkinsonism (1,2), at least in part due to apoptosis (3). There is evidence in some cases of Parkinson's disease that neurons die an apoptotic death (4). Excitotoxicity can also contribute to cell death in Parkinson's disease, as well as other neurodegenerative diseases such as Huntington's disease (5,6).

Scope. The initiation and internal cell signaling of apoptosis is complex. Extracellular cues (binding of FAS ligand to FAS) and intracellular signals (release of cytochrome C from the mitochondria) can initiate or mediate apoptosis. One of the intracellular pathways that transduces apoptosis is c-JUN kinase (JNK). JNK is situated in a vital position to propagate or abrogate apoptosis (7,8). In one experimental paradigm, absence of JNK 3 isoform blocks kainate-dependent apoptosis in the hippocampus (9).

Our hypothesis is that JNK, especially JNK3 isoform, mediates degeneration of substantia nigra neurons after exposure to the neurotoxin MPTP or excitotoxins. Our first strategy is to examine the effects of MPTP in JNK knockout mice on survival of substantia nigra neurons. Progress on this study is presented in this annual report.

Background summary. Several lines of past investigation intersect to provide the underpinning for studies on JNK activity in nejurotoxin dependent Parkinsonism. Neurotoxins can kill neurons through apoptosis (5). The cellular signal transduction of apoptosis can be routed through JNK. Absence of JNK3 nullifies apoptosis in one example of excitotoxicity (kainate induced hippocampal death). Apoptosis has been detected in the substantia nigra in Parkinson's disease (5). In combination, these findings provide support for a role of JNK in understanding neurotoxin induced degeneration of substantia nigra neurons. Thus, if this idea is demonstrated, blocking the activity of a selective JNK isoform would serve to protect against neurotoxin induced apoptosis.

BODY

Studies on the effect of MPTP treatment on survival of substantia nigra neurons will be presented in JNK knockout and wild-type mice.

Experimental methods.

Animals. We used wild-type and JNK knockout mice. Wild-type included mice with 129/B6 strain backgrounds as well as C57BL/6 and SJL/C57BL/6. The JNK 1, 2 and 3 knockout mice are bred in 129/B6 backgrounds. It is known that 129/B6 constitutes a variable genetic strain. We will deal with this issue under "Problems". Animals were at least 6 months old, because MPTP neurotoxicity is most apparent in older mice. Animals were injected with MPTP or vehicle in a glove box in a P3 facility and were kept alive for 7 days before use in immunohistochemistry.

MPTP use. MPTP was injected intraperitoneally. Doses of MPTP ranged from 10 mg/kg for 2 days to 20mg/kg for 5 days. MPTP neurotoxicity was measurable at doses of 40mg/kg as a single injection and 20mg/kg daily for 5 days. Doses of 10mg/kg for 2 days was ineffective. MPTP administration killed the mice at higher doses, such as 20 mg/kg X 4 in one day (80mg total). This dose has been reported previously, but is not practical in our studies in our strains. Pilot studies therefore indicate the total dose of MPTP needs to be at least 40 mg/kg in our mice.

Immunohistochemistry. Animals were anesthetized with Avertin (0.23ml/10g bw ip), perfused with 4% paraformaldehyde and post-fixed for 2 hours. Brain sections (50 um) were cut on a Vibratome, blocked with horse serum and treated with anti-tyrosine monoclonal antibody (Sigma) at a dilution of 1:100. Vectorstain anti-mouse ABC kit was used to detect the diaminobenzidine reaction product. At least 5 sections through the substantia nigra were processed.

Cell counts. We counted tyrosine hydroxylase labeled cells in the substantia nigra, excluding the ventral tegmental area. Counts were made without regard to the treatment or JNK status. Cell counts from both substantia nigras in each animals were added and averaged among the sections.

Statistical analysis. Measurements were provided to the medical school statistician, Dr. Stephan Baker. Tukeys honest square differences test was used.

Results.

Positive findings. The following cell survival was measured.

Animal (N)	treatment	TH-positive cells (SE)
WT (3)	vehicle	118±2
JNK (4)	vehicle	133±2
WT (4)	MPTP	35±2
JNK (10)	MPTP	100±3

Analysis. We used the Tukeys honestly square difference test to evaluate our initial findings. Significant differences were found in comparison of WT MPTP-treated with JNK knockout MPTP-treated ($p=0.016$) and WT vehicle-treated ($p=0.05$).

We also reviewed the distribution and morphology of the substantia nigra neurons, in colloboration with Dr. Marian Difiglia at Massachusetts General Hospital. The MPTP treatment led to a marked reduction in neurons distributed to both the substantia nigra and the ventral tegmental area. Both cells and neuropil (dendrites and axons) in the substantia nigra were sparse (Fig. 1). In contrast, a normal distribution of tyrosine-hydroxylase labeled neurons and neuropil was detected in untreated wild-type mice (Fig. 2) and JNK 1 and 2 knockout mice (Fig. 3). In initial review, we observed that the vehicle-treated animals had no somatic or dendritic morphologic changes in the tyrosine hydroxylase labeled neurons in the substantia nigra. Neurons in the MPTP-treated wild-type mice exhibited shrunken soma and fragmented dendrites. The MPTP-treated JNK1 and JNK2 knockout mice had no evident morphologic changes; however, the MPTP-treated JNK3 knockout mice showed smaller soma and thinned dendrites, despite having surviving neurons at 7 days after MPTP. These initial impressions of single-cell morphological analysis are currently being confirmed.

Negative findings. We found an interesting, but as yet unexplainable, observation. 129/B6 JNK (+/+) littermates of the JNK knockout mice were especially vulnerable to MPTP treatment and frequently died after 2 days. Post-mortem analysis showed no changes in the substantia nigra. In contrast, JNK knockout mice survived the MPTP treatment. We needed to use related strains of mice for wild-type controls. The possible protection of the JNK knockouts to the MPTP effect is therefore noted.

Problems in accomplishing tasks. We are inbreeding the JNK knockouts to C57Bl/6, because the 129/B6 strain is in actuality a variety of substrains and C57Bl/6 can withstand the MPTP treatment to survive 7 days and exhibit substantia nigra neurodegeneration. We are at six generations of inbreeding. According to our consultant, Dr. Stephen Jones in the transgenic core, we can use generation seven mice

for experimentation as essentially C57Bl/6. We also will increase the number of mice studied, to enable our comparison of subtypes of JNK knockouts: JNK 1, 2 and 3.

CONCLUSIONS

Our hypothesis is that JNK mediates degeneration of substantia nigra neurons after treatment with MPTP. Our initial results indicate that JNK might mediate the neurotoxicity of MPTP. In the next year, we expect to decipher the isoform of JNK which relay the MPTP action. Our first year results suggest that JNK 1 and 2 could be more involved in the neurotoxicity than is JNK 3, because of the neuropathological changes observed in the JNK 3 knockout animals. The potential importance of these first results is that blocking JNK could serve to protect against MPTP (and conceivably other) neurotoxicity. To that end, a partial JNK inhibitor, CEP-1347/KT-7515, was reported to block some of the effects of MPTP in the substantia nigra (10).

FIGURE LEGENDS

Figure1. Tyrosine-hydroxylase immunohistochemical labeling in the substantia nigra of MPTP-treated wild-type mice. In this representative example, a wild-type mouse was treated with MPTP at 20mg/kg for 5 days and the brain was processed for immunohistochemistry on day 7. Labeling for tyrosine-hydroxylase was scant in the regional of the substantia nigra (dorsal to the internal capsule). IC denotes internal capsule; arrows point to region of the substantia nigra.

Figure 2. Tyrosine-hydroxylase immunohistochemical labeling in the substantia nigra of an untreated wild-type mouse. Abundant labeling of soma and neuropil was detected in wild-type mice. IC denotes internal capsule; arrows point to region of the substantia nigra.

Figure 3. Tyrosine-hydroxylase immunohistochemical labeling in the substantia nigra of MPTP-treated (20mg./kg x 5d) JNK 1 knockout mice. In this representative example, the MPTP treatment did not change the distribution of TH-positive labeling in the substantia nigra (situated above the internal capsule) or the ventral tegmental area (medial to the substantia nigra). TH-positive labeling was identified in neurons and neuropil. IC denotes internal capsule; arrows point to region of the substantia nigra.

Detailed analysis of morphological changes at the single cell level is currently being undertaken.

Figures

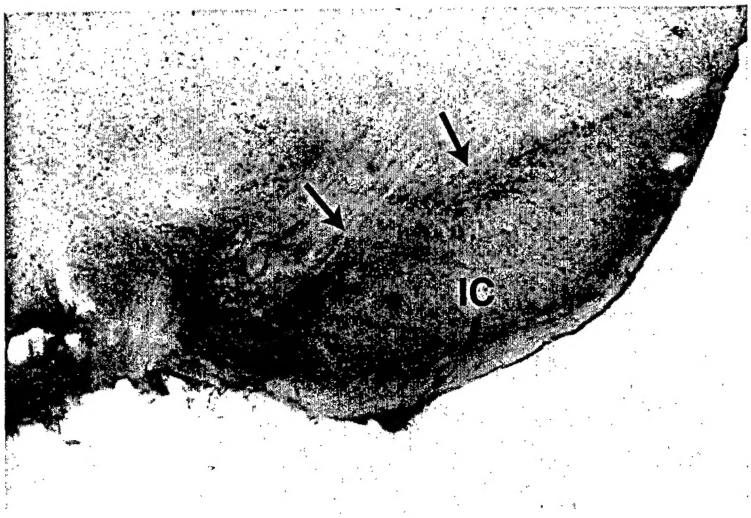

Fig. 1

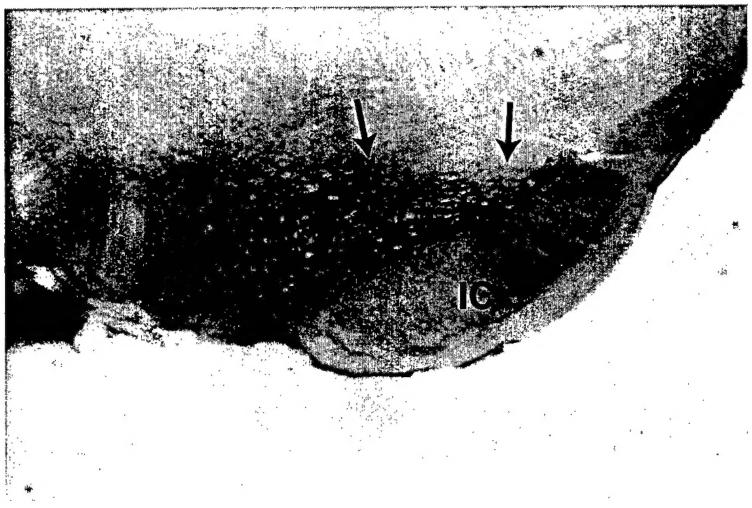

Fig. 2

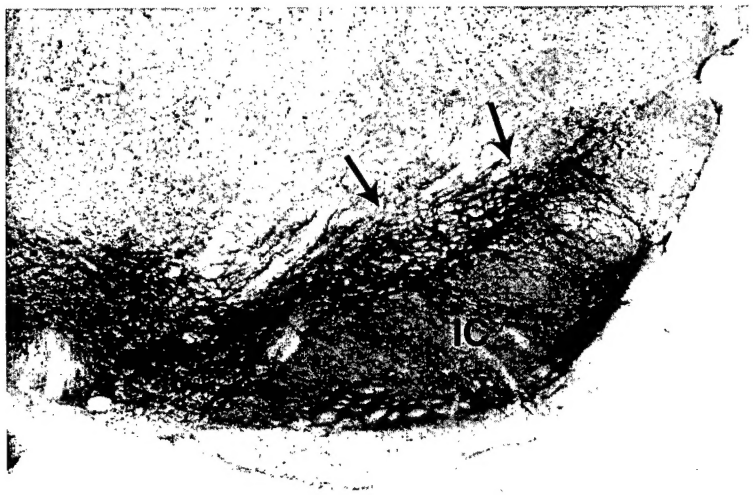

Fig. 3

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November 22, 1999

Neil Aronin, M.D.
Professor of Medicine and Cell Biology
University of Massachusetts Medical School
55 Lake Avenue North
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Dear Neil:

It is my pleasure to provide this letter of support and counsel for your continued use of our Transgenic/Knockout Animal Core Facility at the University of Massachusetts Medical School.

The JNK knockout colonies are currently in excellent health. When we initially received the JNK mice from an outside university, the colonies had a virus infection and needed to be kept in quarantine for many months. I realize that the quarantine delayed the start of your experiments, but the Core Facility and the Department of Animal Medicine felt that this action was necessary to protect the vivarium. The colony became free of virus early in 1999 and was transferred to our specific-pathogen free facility. We continue to monitor these colonies frequently for the presence of pathogens.

The initial studies in JNK knockout mice on the protection of the substantia nigra after MPTP treatment are quite exciting. I hope to continue our regular consultations (as we have done for the past year) for maintenance of the colony and the breeding and development of subsequent mouse lineages.

Sincerely,

Stephen Jones, Ph.D.
Assistant Professor of Cell Biology
and Program in Genetics
Director, Transgenic/Knockout Animal Core Facility
University of Massachusetts Medical School